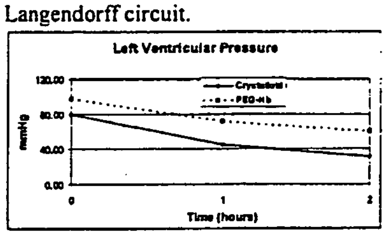
APPENDIX 1

CARDIAC FUNCTION AFTER 8-HOUR STORAGE USING PEG-HEMOGLOBIN VS. CRYSTALLOID PERFUSION

Efforts to extend myocardial preservation for transplantation by crystalloid perfusion has been limited by edema and compromised function. We hypothesized that hypothermic perfusion preservation with an oxygen carrying hemoglobin solution may extend preservation times. The purpose of this study was to compare cardiac function after continuous perfusion with PEG-Hemoglobin (Hb) vs. a physiologic crystalloid perfusate. The hearts of 9 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=4) hearts were continuously perfused with PEG-Hb (Enzon Inc., N.J.) at 20°C and 30 mmHg for 8 hours. Group II (n=5) hearts were continuously perfused with crystalloid perfusion for 8 hours at 20°C. Cardiac function was measured with a left ventricular balloon at 0, 1, and 2 hours after transfer to a standard crystalloid Langendorff circuit.



Heart rate was the same for group I and II through the testing period (89.6 vs. 91.1, p=0.57). Developed LV pressure (systolic minus diastolic) at 0.6cc LV volume was greater in Group I (76.17±19.2 mmHg), than in Group II (52.0±25.21, p=0.021). Maximum +dP/dT at 0.6 cc LV volume was greater in Group I (854.47±381.8mmHg/sec) than in Group II (485.10±284.14 mmHg/sec, p=0.025). Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II. Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 20°C yields left ventricular function is superior to 8-hr crystalloid perfusion. despite similar myocardial edema. Extended cardiac perfusion preservation with PEG-Hb may prove to be useful in transplantation.

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24 Hour Cardiac Perfusion Preservation Using a Novel PEG-Hemoglobin Solution

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Cardiac preservation for transplantation is limited by ischemic hypothermic storage of 4 to 6 hours. Hypothermic perfusion preservation using a novel oxygen carrying hemoglobin solution may extend preservation times and decrease ischemic injury. The purpose of this study was to compare cardiac function after 24 hrs of continuous hypothermic perfusion with a PEG-Hemoglobin (Hb) solution to the clinical standard of hypothermic ischemic preservation.
Methods: The hearts of 25 anesthetized and intubuted NZW rabbits were harvested after cold cardioplegic arrest. Group I(n=7) hearts were perfused with a PEG-Hb solution at 20C and 30 mmHg for 24 hours. Group II(n=10) hearts were preserved by cold ischemic storage for 4 hours at 4C, and Group III(n=8) were tested immediately after harvest. LV function was measured in the non-working state immediately and 2 hours after transfer to a standard crystalloid Langendorff circuit.
Results: Developed LV pressure at 0.5cc LV volume was similar in Group I (54.2 ±2.6mmHg) and Group II (49.1±5.4mmHg,p=.5) but greater in Group III (69.4±5.1mmHg, p=.02). Maximum-dp/dT at 0.5 cc LV volume was similar in Group I(-398.1±19.0 mmHg/sec), Group II (-354.8±49.1mmHg/sec,p=.2) and Group III(-456.2±44.1mmHg/sec,p=.7). Maximum +dp/dT at 0.5cc LV volume was also similar in Group I (660.3±49.5mmHg/sec), Group II (428.4±54.9mmHg/sec,p=.3) and Group III(514.6±48.9mmHg/sec,p=.6).
Conclusions: Continuous perfusion preservation of rabbit hearts for 24 hrs with this novel PEG-Hb solution at 30 mmHg and 20 C yields left ventricular function that is similar to 4 hrs of ischemic hypothermic storage and to that of fresh control hearts. Extended cardiac perfusion preservation with this PEG-Hb solution deserves further investigation in large animal transplant models.

APPENDIX 2

PERFUSION PRESERVATION WITH NOVEL PEG-HB SOLUTION MAY ALLOW RECOVERY OF FUNCTION IN THE DONOR RABBIT CARDIAC ALLOGRAFT AFTER CARDIOPLEGIC ARREST.

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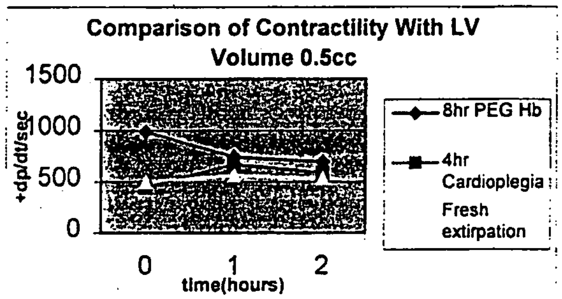
INTRODUCTION: The clinical standard of hypothermic storage preservation of the cardiac allograft imposes an ischemic insult to the allograft and limits preservation of the allograft to 4 to 6 hours. Continuous hypothermic perfusion preservation with a novel, oxygen carrying, PEG-hemoglobin (Hb) solution may decrease ischemic injury and allow better and lengthier preservation of cardiac function than the clinical standard. The purpose of this study was to compare cardiac function after 8 hrs of continuous hypothermic perfusion using a novel PEG-Hb solution to 4 hrs of hypothermic ischemic storage preservation.

METHODS: The hearts of 28 anesthetized and intubated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=10) hearts were continuously perfused via the aortic root with a novel PEG-Hb solution at 20°C and 30 mmHg for 8 hours. PO_2 was maintained greater than 500 mmHg during the preservation phase. Group II (n=10) hearts were preserved by cold ischemic storage for 4 hours at 4°C. Group III hearts (n=8) were tested immediately after their harvest. Left ventricular function was measured at 37°C in the non-working state 15 min after transfer to a standard crystalloid Langendorff circuit.

RESULTS: Developed LV pressure at 0.5cc LV volume was greater in Group I (75.7±10.3mmHg) than Group II (49.1±5.4mmHg,p=.04) and similar to Group III (69.4±5.1mmHg, p=.6). Maximum-dp/dT at 0.5 cc LV volume was greater in Group I(-610.6±68.4mmHg/sec) than Group II (-354.8±49.1mmHg/sec,p=.01) and trended toward superiority over Group III(-456.2±44.1mmHg/sec,p=.09). Maximum +dp/dT at 0.5cc LV volume was greater in Group I (964.9±156.6mmHg/sec) than both Group II (428.4±54.9mmHg/sec,p=.004) and Group II (514.6±48.9mmHg/sec, p=.02).



CONCLUSIONS: Continuous perfusion preservation of the rabbit heart for 8 hrs with this PEG-Hb solution at 30 mmHg and 20°C yields left ventricular function that is superior to 4 hrs of ischemic hypothermic storage. Furthermore, return of cardiac function after perfusion preservation using this PEG-Hb solution may be superior to that obtained in freshly arrested hearts. These data suggest there may occur some recovery of myocardial function during perfusion preservation with this PEG-Hb solution after the ischemic insult of cardioplegic arrest. Perfusion preservation using this PEG-Hemoglobin solution may also be more useful than hypothermic ischemic storage in the reanimation of non-beating heart donors. Continuous perfusion preservation using this PEG-Hb solution deserves further investigation in large animal transplant models.

APPENDIX 3

CARDIAC FUNCTION AFTER 8-HOUR STORAGE USING PEG-HEMOGLOBIN VS. CRYSTALLOID PERFUSION

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ABSTRACT

Introduction: Efforts to extend myocardial preservation for transplantation by crystalloid perfusion have been limited by edema and compromised function. We hypothesized that hypothermic perfusion preservation with a polyethylene glycol (PEG) conjugated hemoglobin solution may extend preservation times. The purpose of this study was to compare cardiac function after continuous perfusion using a hypocalcemic normokalemic crystalloid perfusate with and without the addition of PEG-Hemoglobin (Hb).

Methods: The hearts of 20 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=10) hearts were continuously perfused with a hypocalcemic, normokalemic 3% bovine PEG-Hb solution at 20°C and 30 mmHg for 8 hours. Group II (n=10) hearts were continuously perfused with an identical crystalloid solution without PEG-Hb for 8 hours under the same conditions as Group I hearts. Cardiac function was measured with a left ventricular force transducer after transfer to a standard crystalloid Langendorff circuit at 37 °C and an aortic root pressure of 59 mmHg.

Results: After 8 hours of perfusion preservation, heart rate was similar for groups I and II (p=NS). Coronary blood flow after preservation and during preservation was similar between PEG-Hb and crystalloid preserved hearts (p=NS). Left ventricular developed pressure, peak dP/dt, and peak $-dP/dt$ were superior in hearts preserved with PEG-Hb. Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II (p=NS).

Conclusions: Continuous perfusion preservation of rabbit hearts for 8 hours with a hypocalcemic normokalemic PEG-Hb based solution at 30 mmHg and 20°C yields left ventricular function that is superior to perfusion a similar crystalloid solution without PEG-Hb, despite similar myocardial edema and coronary flow. Extended cardiac perfusion preservation with this PEG-Hb

based solution deserves further study including comparison to traditional cardioplegic preservation solutions.

INTRODUCTION

The current method of donor heart preservation for clinical transplantation involves cold cardioplegic arrest and storage at near freezing temperatures. Because of ongoing ischemia, this preservation technique prohibits extended storage of donor organs, use of advanced methods of tissue typing, and delivery of donor hearts over long distances. The current preservation technique may also lead to irreversible graft damage. Preservation by continuous coronary artery perfusion allows for greater preservation times than hypothermic ischemic preservation (1). Continuous coronary artery perfusion allows for an ongoing supply of substrate as well as removal of metabolic waste products. Three general types of solution have been examined for their efficacy as cardiac preservation agents. Perfusion with crystalloid, cardioplegia-type solutions have shown limited promise (2-6). Perfusion preservation using these solutions has been limited by edema and compromised cardiac function (2-6). Similarly, studies examining perfluorocarbon emulsions as perfusion preservation media for the donor heart have produced mixed results (1,7-10). Further, perfluorochemicals are expensive and have questionable safety profiles when used systemically (7-9).

Hemoglobin-based blood substitutes have more recently been developed for use as blood replacements in trauma and surgery. Use of these solutions as organ preservation solutions may lengthen the window of *ex vivo* cardiac preservation with a concomitant decrease in ischemia. We hypothesized that hypothermic perfusion preservation with a hypocalcemic, normokalemic, polyethylene glycol conjugated hemoglobin (PEG-Hb) based solution over 8 hours would preserve left ventricular function above that obtained with a chemically identical crystalloid

solution without PEG-Hb. We suspect that this PEG-Hb solution may optimize the donor heart during its preservation period.

The purpose of this study is to compare *ex vivo* adult rabbit heart preservation after continuous coronary artery perfusion using a hypocalcemic, normokalemic PEG-Hb solution versus an identical hypocalcemic, normokalemic crystalloid solution not containing PEG-Hb. This work will lay the foundation for future investigation comparing perfusion preservation with PEG-Hb based solutions to hypothermic ischemic storage preservation using traditional cardioplegic solutions as well as PEG-Hb solutions containing specific enhancers of myocardial preservation.

METHODS

All animals received humane care in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 86-23). The Institutional Animal Care and Use Committee of the University of California, Irvine, approved animal procedures.

Experimental Design

The hearts of 20 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=10) hearts were continuously perfused with a normokalemic hypocalcemic bovine PEG-Hb based solution at 20°C and 30 mmHg of aortic root pressure for 8 hours. Group II (n=10) hearts were identically preserved with a crystalloid solution identical in composition to Group I hearts, but without the addition of PEG-Hb.

Cardiac Procurement

Twenty adult male New Zealand White rabbits (3 to 3.5 kg) were anesthetized using an intramuscular injection of 50mg ketamine and 5mg xylazine per kilogram. Lactated Ringers

solution was infused through an IV catheter in a marginal ear vein at a rate of 5 to 15 cc/hr. The rabbits were mechanically ventilated using a Servo Animal Ventilator (model #900C, Siemens-Elema, Sweden). Anesthesia was maintained with intravenous ketamine/xylazine in a 1:1 ratio. A median sternotomy followed by a longitudinal pericardial incision was performed, exposing the heart and mediastinal vessels. All rabbits received 1,000 U heparin sodium/kg IV. The innominate artery, the aortic arch between the brachiocephalic trunk and left carotid artery, as well as the inferior and superior vena cava were the identified and isolated. Upon ligation of the inferior and superior vena cava, the innominate artery was cannulated using an 18 Ga angiocatheter. 60 cc of hypothermic cardioplegia solution (2-4°C) was administered to the coronary arteries via the innominate artery over 3 minutes. An arteriotomy was made in the pulmonary trunk to decompress the right ventricle. Hypothermic normal saline (2-4°C) was used to cool the heart during cardioplegia infusion. The heart was quickly excised and placed in cold saline (4°C) for further dissection. The heart was trimmed of excess soft tissue including lungs, trachea, and thymus. All hearts were placed onto the preservation circuit by cannulation at the ascending aorta. Coronary perfusion was begun within 5 minutes of cardiectomy. All hearts were preserved for 8 hours by continuous coronary artery perfusion. Aortic root pressure was maintained at 30 mmHg. Temperature of the perfusate was maintained at 20°C. All hearts were perfused and immersed in the respective preservation solutions for the entire 8-hour preservation period. 95%O_2/5%CO_2 administration was begun using the membrane oxygenator 15 minutes after transfer of the heart to the preservation circuit. PaO_2 was maintained at a level greater than or equal to 600mHg. The preservation circuit consisted of a centrifugal pump (Medtronic Bio-Medicus pumphead, Model # 9154R, Medtronic Blood Systems, Inc., Anaheim, CA) and Bio-Console (Medtronic Bio-Medicus Inc., Eden Prairie, MN), an adult membrane oxygenator

(Sarns/3M Health Care, Inc., Ann Arbor, MI), C-Flex Consolidated Polymer tubing (Fischer, Largo, FL), a 40 um blood filter (Pall Biomedical, Inc, Fajardo, PR), and 2 glass reservoirs. The temperature of perfusate was maintained by a heater/cooler (Fisher Scientific Inc., Pittsburgh, PA) which was circulated through the membrane oxygenator (figure 1).

Preservation Solutions

The composition of the PEG-Hb based preservation fluids is as follows: 3% bovine PEG-Hb, KCL (4.7 mEq/L), NaCl (148.7 mmol/L), NaH2PO4 (2.5 mmol/L), NaHCO3 (2.5 mmol/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6units/L), Tromethamine (THAM) solution (7.3 cc/L). The osmolality of the 3% PEG-Hb solution is 324 mOsm/kg.

The composition of the crystalloid preservation solution is as follows: KCL (4.7 mEq/L), NaCl (150.7 mEq/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6 units/L), and Tromethamine (THAM) solution (7.3 cc/L). The osmolality of the crystalloid preservation solution is 324 mOsm/kg.

Postpreservation Assessment of Cardiac Function:

At the end of the 8-hour preservation period, all hearts were transferred to an isolated heart perfusion apparatus for purposes of data collection. Coronary perfusion via the aortic root was immediately begun at 37°C and 59 mmHg aortic root pressure. 95%O_2/5%CO_2 administration was begun using the membrane oxygenator 15 minutes after transfer of the heart to this circuit. PaO_2 was maintained at a level greater than or equal to 600mHg. After 15 minutes

of coronary perfusion in this position, coronary flow, heart rate, left ventricular developed pressure (LVP), peak dP/d*t*, and peak –dP/d*t* were measured. Coronary flow and heart rate were measured every 15 minutes for 2 hours. LVP, peak dP/d*t*, and peak –dP/d*t* were measured again at 75 and 135 minutes following transfer to the second circuit. Heart rate was measured by counting left ventricular contractions over the course of one minute. Coronary flow was measured by collecting the effluent that exited from the pulmonary artery over course of one minute. LVP, peak dP/d*t*, and peak –dP/d*t* were measured in the beating, nonworking position during continuous coronary artery perfusion. Developed left ventricular pressure (systolic minus diastolic) and peak rates of left ventricular pressure development (dP/dt_{max}) and relaxation (-dP/dt_{max}) were measured using a left ventricular force transducer (Biopac Systems, Inc., Santa Barbara, CA). Data from the LV force transducer was digitized using an analog to digital converter (Biopac Systems, Inc., Santa Barbara, CA) and analyzed using Acknowledge software (Version 3.2.6, Biopac Systems, Inc., Santa Barbara, CA) and a desktop computer (Nexstar, Fremont, CA). The testing circuit consisted of a centrifugal pump (Medtronic Bio-Medicus pumphead, Model # 9154R, Medtronic Blood Systems, Inc., Anaheim, CA) and Bio-Console (Medtronic Bio-Medicus Inc., Eden Prairie, MN), an adult membrane oxygenator (Sarns/3M Health Care, Inc., Ann Arbor, MI), C-Flex Consolidated Polymer tubing (Fischer, Largo, FL), a 40 um blood filter (Pall Biomedical, Inc, Fajardo, PR), and 2 glass reservoirs. The temperature of perfusate was maintained by a heater/cooler (Fisher Scientific Inc., Pittsburgh, PA), which was circulated through the membrane oxygenator.

Testing Solution: Left ventricular function was assessed in all hearts in both groups using a standard physiologic crystalloid solution. The composition was as follows: KCL (4.7 mEq/L), NaCl (151.5 mEq/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (2.0 mEq/L), lidocaine HCl (12.5 mg/L),

heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human insulin (30.7 units/L), and Tromethamine (THAM) solution (6.1 cc/L).

Measurement of ventricular water content: After 135 minutes of retrograde aortic perfusion on the testing circuit, the ventricular myocardium of the initial 5 hearts in each group was dissected free of atria and other soft tissue. The left ventricular myocardium was weighed before and after desiccation at 110°C.

Data Analysis

Data are reported as means ± SE. Statistical analysis was performed using Systal 7.0.1 software package (SPSS, Inc., Chicago, IL). The Student's t-test was used to test for significance between groups. A p value of less than 0.05 was considered significant.

Materials

Bovine PEG-Hb was obtained from Enzon, Inc. (Piscataway, NJ) in a solution containing 6% PEG-Hb, 5 mM NaH2PO4, 5 mM NaHCO3, and 150 mM NaCl. Polyethylene glycol (PEG) conjugated bovine Hb (PEG-Hb) was prepared by the isolation of hemoglobin from bovine red blood cells obtained from a closed herd. The material was purified and each Hb molecule modified with approximately 12 succinimidyl carbonate polyethylene glycol strands (5000 daltons) to yield a 6% (g/dL) Hb solution with methemoglobin less than 5% of total hemoglobin, endotoxin less than 0.5 EU/mL, and viscosity 3.1 cP at 37°C. Normal saline solution (0.9% NaCl) was obtained from Baxter Health Care (Irvine, CA). Solutions were monitored using a blood gas analyzer (288 Blood Gas System, Ciba-Corning Diagnostics Corp., Medfield, MA), an Automated Coagulation Timer (Medtronic Hemotec, Inc., Englewood, CO) and a blood glucose meter (Lifescan, Inc., Milpitas, CA). Membrane oxygenators were obtained from Sarns/3M Health Care, Inc. (Ann Arbor, MI).

RESULTS

Developed LV pressure: Developed LV pressure at 0.5 cc LV volume was similar between PEG-Hb and crystalloid preserved hearts at 15 minutes after the end of preservation (p=0.46, figure 2). However, developed LV pressure at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 75 (p=0.006) and 135 minutes (p=0.002) after the end of preservation.

Maximum rate of LV contraction: Peak dP/dt_{max} at 0.5 cc LV volume trended toward superiority amongst hearts preserved using PEG-Hb solution compared to crystalloid preserved hearts, at 15 minutes after the end of preservation (p=0.10, figure 3). However, peak dP/dt_{max} at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 75 (p=0.01) and 135 minutes (p=0.001) after the end of preservation.

Maximum rate of LV relaxation: Peak $-dP/dt_{max}$ at 0.5 cc LV volume was similar between PEG-Hb and crystalloid preserved hearts at 15 minutes (p=0.27) after the end of preservation and trended toward superiority at 75 minutes after the end of preservation (p=0.07, figure 4). Peak $-dP/dt_{max}$ at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 135 minutes (p=0.006) after the end of preservation.

Ventricular Water Content: Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II (p=NS).

Coronary Flow: Coronary flow after preservation was similar between PEG-Hb and crystalloid preserved hearts.

Heart Rate: Heart rate was the same for group I and II through the testing period (p=NS, Table 1).

CONCLUSIONS

There are two general techniques of *ex vivo* cardiac preservation for transplantation. The standard of care and commonly used technique is hypothermic ischemic immersion storage of the donor cardiac allograft. The second method of cardiac preservation is coronary perfusion preservation. These two methods can and have been used in combination with improved results. Perfusion preservation is superior to immersion preservation because it allows for the continuous washout of metabolic waste products, as well as the delivery of nutrients, metabolic substrate, and oxygen to the myocardium (1). For several reasons, perfusion preservation has not been applied clinically to *ex vivo* cardiac preservation for transplantation. First, a user friendly, practical, and portable perfusion preservation device is not currently available. Second, most research into perfusion preservation to date has been performed using crystalloid cardioplegia solutions and perfluorocarbons. Crystalloid cardioplegia solutions, unfortunately, carry very little oxygen and hence their use is associated with considerable ischemic injury to the donor organ (2-6). Perfluorocarbon based solutions have demonstrated mixed results for the purpose of cardiac preservation and are extremely expensive (1, 7-10).

Perfusion preservation using stroma-free hemoglobin based solutions represents an innovative means of *ex vivo* cardiac preservation. Stroma-free hemoglobins were initially developed as blood substitutes for use in the treatment of life threatening hemorrhage secondary to trauma. There is strong interest among transplant scientists in the potential for these solutions as organ preservation solutions. The purpose of this study was to assess the utility of perfusion preservation using normokalemic hypocalcemic polyethylene glycol coated bovine hemoglobin based solution.

The superior organ preservation results of the PEG-Hb preserved hearts in this study are probably a result of a combination of both an oncotic and oxygen delivery effect of PEG-Hb. Data supporting an oxygen delivery effect of PEG-Hb has otherwise been obtained using exchange-transfusion in a rat model (11). Rats were exchange-transfused up to an 85% hematocrit reduction with either PEG-Hb, PEG-mHb (50%-methemoglobin), PEG-carbon monoxide hemoglobin (PEG-COHb), or PEG-human serum albumin (PEG-HSA). Survival at twenty-four hours after transfusion was 79 % in the PEG-Hb group, 30 % in the PEG-mHb group, and 0% for both PEG-COHb and PEG-HSA. Despite similar plasma expansion properties of the 4 solutions, the solution with greatest oxygen delivery capability led to greatest survival.

On a per gram basis, the oxygen carrying capacity of PEG-Hb is the same as would be found with unmodified tetrameric bovine Hb. PEGylation of Hb involves the covalent attachment of polyethylene glycol to stroma-free Hb tetramers. PEGylation does not appear to change the total oxygen carrying capacity of the Hb, but PEGylation does appear to alter the nature of oxygen transport (12). For example, because of its larger particle size, PEG-Hb remains within the vascular space for longer than otherwise unmodified Hb (13). In addition, PEGylation alters the oxygen affinity of bovine hemoglobin. The P_{50} of bovine PEG-Hb is 15 torr at 37°C (14). Clearly, this is a relatively low P_{50}. Such high oxygen affinity begs the question of the ability of PEG-Hb to effectively deliver oxygen under normothermic and hypothermic conditions. Bovine PEG-Hb has been shown using the rat model to provide better tissue oxygenation than stroma-free bovine Hb (P_{50} – 26 torr) or cross-linked bovine Hb (P_{50} – 48 torr), both of which have lower affinity for oxygen than does PEG-Hb and therefore should theoretically be better tissue oxygenators (14). Furthermore, we also know that bovine Hb is unlike human Hb in that it does not require 2,3-diphosphoglycerate to lower its oxygen affinity,

but rather requires only chloride ions, which are present in the PEG-Hb preservation solution (15). Finally, the Bohr effect is more pronounced in bovine Hb than human Hb, which would theoretically allow better delivery of oxygen at lower pH and temperature (16).

The oncotic pressure of PEG-Hb is greatly enhanced by the conjugation of PEG to surface amino acid groups of the Hb. A 3 gm/dL solution, as used in this study, has a colloid osmotic pressure of approximately 39 mm Hg (17). In comparison, similar concentrations of human serum albumin and purified human hemoglobin A_0 have colloid osmotic pressures of 9 mm Hg and 9 mm Hg, respectively (17). The amount of human serum albumin used in both preservation solutions in this study, 0.15 gm/dL, has an oncotic pressure on the order of 1 mm Hg (17). The average calculated molecular weight for unmodified and intramolecularly cross-linked human tetramers is 65,300 ± 3500 compared to 117,000 for bovine PEG-Hb. When added to Bretschneider's HTK cardioplegic solution, PEG is associated with improved recovery of left ventricular function as well as less myocardial edema (18), and it is likely that the onconicity of the PEG solution plays an important role. The mechanism of action of PEG may also involve suppression of lipid peroxidation (18).

The preservation solution was made hypocalcemic because the intracellular accumulation of calcium during ischemia and reperfusion is associated with cellular injury (19-23) and a hypoxically stressed heart may be protected by a hypocalcemic solution (19). The solution was normokalemic in order to keep the heart beating, since a beating heart may be less susceptible to edema. Finally, the preservation solution was slightly hypermagnesemic because magnesium inhibits the membrane transport of calcium, and thus intracellular accumulation of calcium, which should help to prevent the deleterious effects of calcium (24-27). Magnesium has been

shown to attenuate deleterious effects of calcium in ischemic piglet hearts, which are more sensitive to the detrimental effects of calcium than are adult hearts (28).

There is tremendous value to lengthening the window of cardiac preservation. First, less ischemia to the donor organ will likely improve posttransplant graft function and recipient survival. Second, lengthening the window of cardiac preservation will allow prospective HLA matching as well as the transport of hearts over greater distances to better matched recipients.

Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 20^0C yields left ventricular function that is superior to 8-hr perfusion with a chemically similar crystalloid solution without addition of PEG-Hb, despite similar myocardial edema. This study addresses myocardial performance following perfusion with and without the PEG-hemoglobin oxygen carrier, since the control group does not really represent an alternate myocardial preservation scheme. Similarly, the mechanism of preservation using this PEG-Hb solution may or may not involve enhanced oxygen delivery. Extended cardiac perfusion preservation with this PEG-Hb based solution deserves further study, including comparison to traditional cardioplegic preservation solutions. It is possible that PEG-Hb may be a useful component of a future clinical preservation solution.

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LEGEND

Figure 1. Isolated heart perfusion preservation circuit.

Figure 2. Developed LV pressure at 15, 75, and 135 minutes after preservation. The Student's t-test was used to test for significance between groups. A p value of less than 0.05 was considered significant (*).

Figure 3. Maximum rate of LV contraction at 15, 75, and 135 minutes after preservation. The Student's t-test was used to test for significance between groups. A p value of less than 0.05 was considered significant (*).

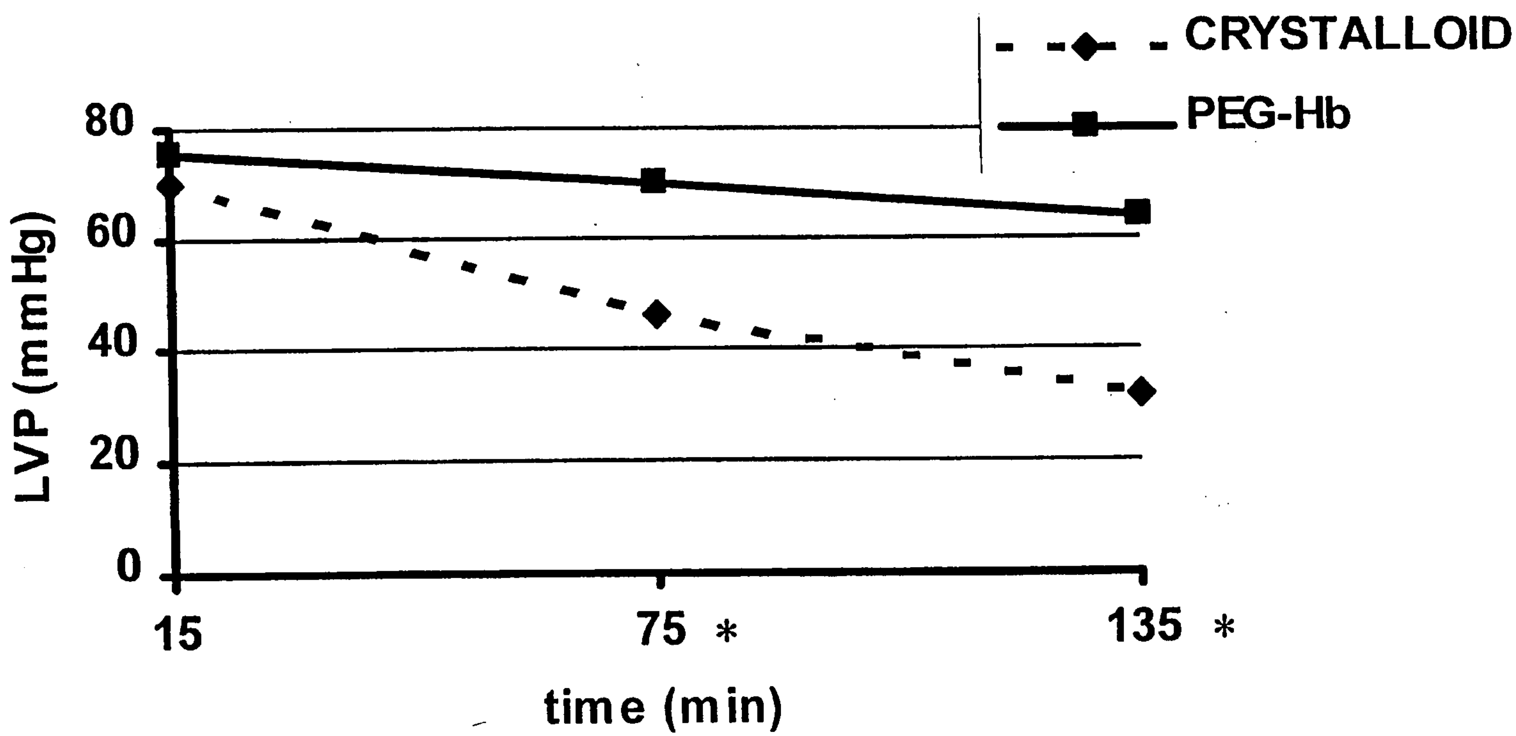
Figure 4. Maximum rate of LV relaxation at 15, 75, and 135 minutes after preservation. The Student's t-test was used to test for significance between groups. A p value of less than 0.05 was considered significant (*).

Table 1. Heart rate after 8 hours of perfusion preservation.

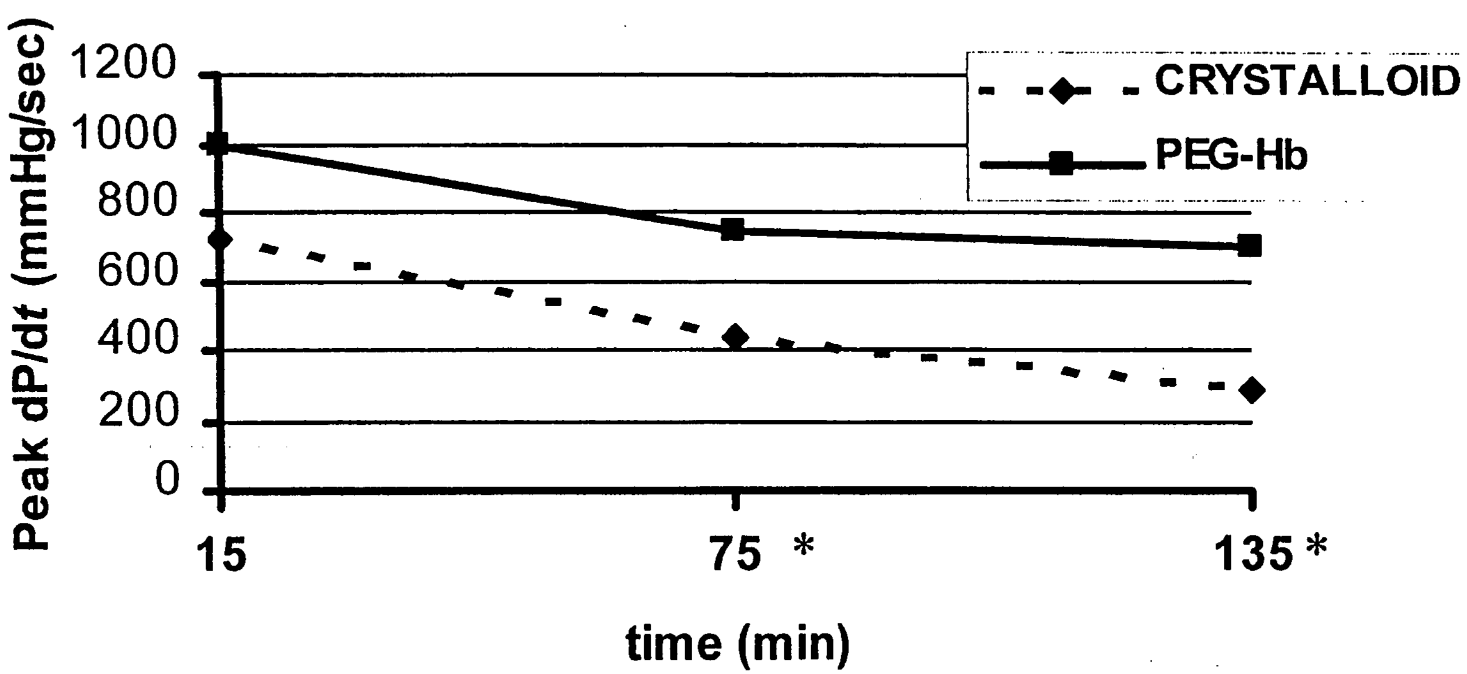
	Heart Rate		
Time (minutes)	PEG-Hb	Crystalloid	p value
15	117±8.4	100.8±8.4	0.19
30	104.9±7.9	98.4±4.9	0.48
45	98.8±6.6	95.2±4.6	0.66
60	94.1±6.6	97.6±5.7	0.70
75	90.9±6.8	96.2±5.5	0.55
90	99.2±6.6	97.0±3.5	0.77
105	97.8±6.3	89.1±4.8	0.30
120	95.0±6.4	75.8±11.5	0.14
135	100.5±6.3	73.9±13.7	0.07

Table 1.

Developed LV Pressure

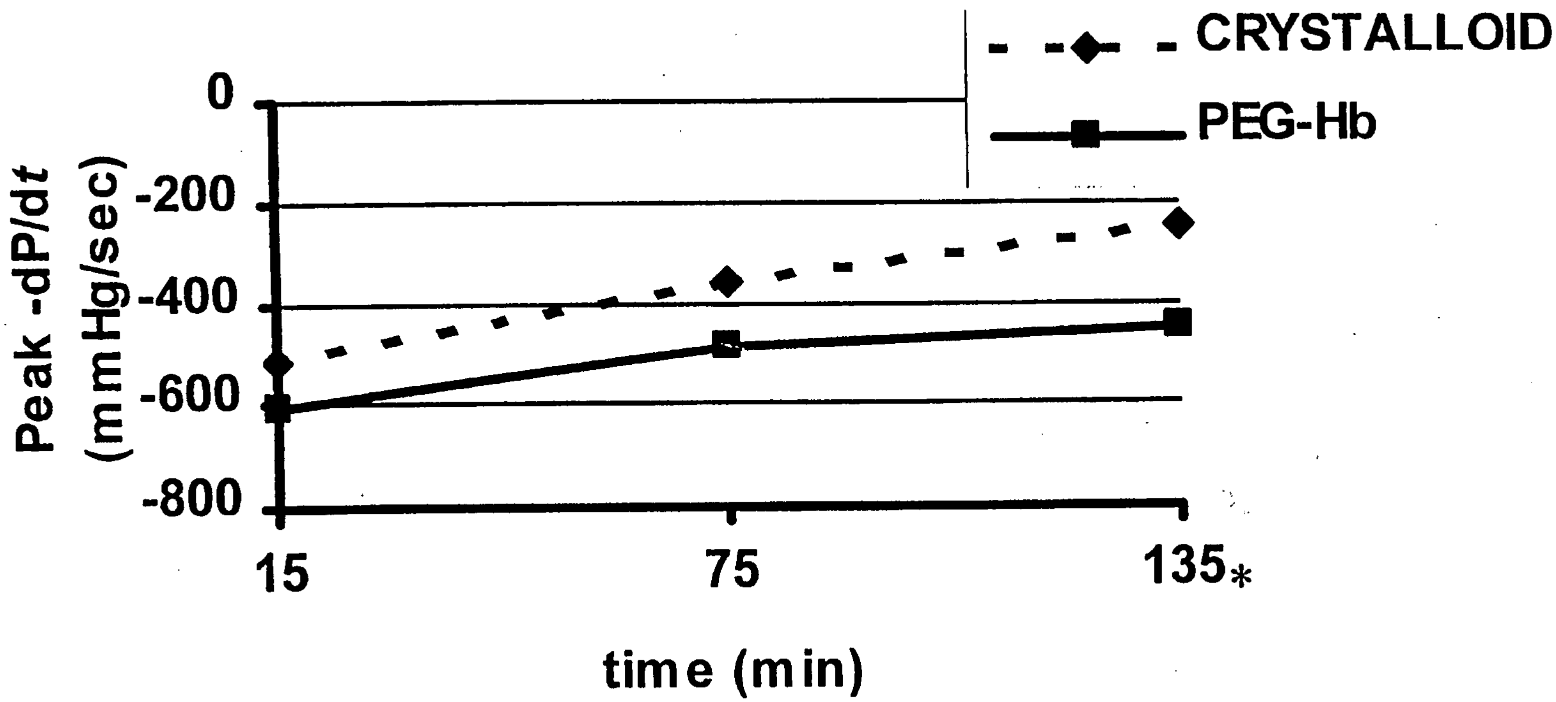


Peak dP/dt



Peak dP/dt (mmHg/sec)
1200
1000
800
600
400
200
0
15
75 *
135 *
time (min)
CRYSTALLOID
PEG-Hb

Peak -dP/d*t*


CRYSTALLOID
PEG-Hb
Peak -dP/dt
(mmHg/sec)
0
-200
-400
-600
-800
15
75
135*
time (min)

APPENDIX 4

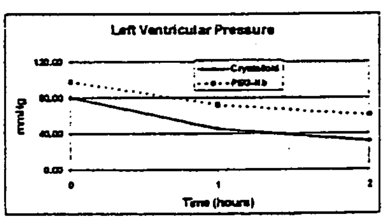
CARDIAC FUNCTION AFTER 8-HOUR PERFUSION PRESERVATION USING PEG-HEMOGLOBIN

Introduction: Efforts to extend myocardial preservation for transplantation by crystalloid perfusion has been limited by edema and compromised function. We hypothesized that hypothermic perfusion preservation with an oxygen carrying hemoglobin solution may extend preservation times.

Preclinical Study 1:
The purpose of this study was to compare cardiac function after continuous perfusion with PEG-Hemoglobin (Hb) vs. a physiologic crystalloid perfusate.

Methods: The hearts of 9 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=4) hearts were continuously perfused with PEG-Hb (Enzon Inc., N.J.) at 20^0C and 30 mmHg for 8 hours. Group II (n=5) hearts were continuously perfused with crystalloid perfusion for 8 hours at 20^0C and 30 mmHg. To both solutions were added KCl (4 mmol/L), Na^+ (145 mmol/L), $MgSO_4$ (5.1 mmol/L), $CaCl_2$ (0.4 mmol/L), 12.5 mg/L lidocaine, heparin (1250 units/L), dextrose (1.25 gm/L), human albumin (1.6 gm/L), human insulin (3.1 units/L). PO_2 was maintained greater than 500 mmHg, and pH of 7.1 (37°C). Cardiac function was measured with a left ventricular balloon at 0, 1, and 2 hours after transfer to a standard crystalloid Langendorff circuit.

Results: Heart rate was the same for group I and II through the testing period (89.6 vs. 91.1, $p=0.57$). Developed LV pressure (systolic minus diastolic) at 0.6cc LV volume was greater in Group I (76.17±19.2 mmHg), than in Group II (52.0±25.21, $p=0.021$). Maximum +dP/dT at 0.6 cc LV volume was greater in Group I (854.47±381.8mmHg/sec) than in Group II (485.10±284.14 mmHg/sec, $p=0.025$). Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II.



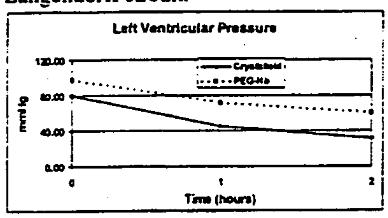
Attachment 2

Conclusions: Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 20^0C yields left ventricular function is superior to 8-hr crystalloid perfusion, despite similar myocardial edema. Extended cardiac perfusion preservation with PEG-Hb may prove to be useful in transplantation.

Preclinical Study 2:
Methods: The hearts of 14 anesthetized and intubated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=4) hearts were perfused with PEG-Hb (Enzon Inc., N.J.) at 20^0C and 30 mmHg for 8 hours. Group II (n=5) hearts were preserved without perfusion for 4 hours at 4^0C, and Group III (n=5) were tested immediately upon harvest.

CARDIAC FUNCTION AFTER 8-HOUR STORAGE USING P
HEMOGLOBIN VS. CRYSTALLOID PERFUSION

Efforts to extend myocardial preservation for transplantation crystalloid perfusion has been limited by edema and compromised funct We hypothesized that hypothermic perfusion preservation with an oxy carrying hemoglobin solution may extend preservation times. The purp of this study was to compare cardiac function after continuous perfu with PEG-Hemoglobin (Hb) vs. a physiologic crystalloid perfusate. hearts of 9 anesthetized and ventilated NZW rabbits were harvested a cold cardioplegic arrest. Group I (n=4) hearts were continuously perfu with PEG-Hb (Enzon Inc., N.J.) at 20°C and 30 mmHg for 8 hours. Gr II (n=5) hearts were continuously perfused with crystalloid perfusion f hours at 20°C. Cardiac function was measured with a left ventric balloon at 0, 1, and 2 hours after transfer to a standard crystal Langendorff circuit.

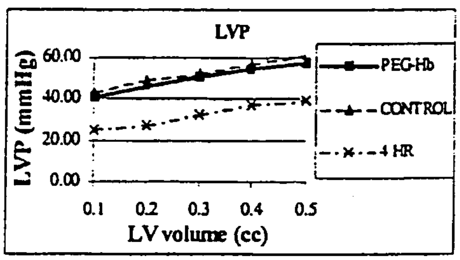


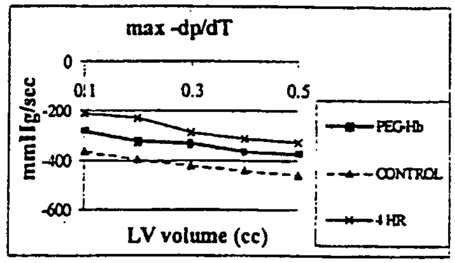
Heart rate was the same group I and II through testing period (89.6 vs. 91 p=0.57). Developed pressure (systolic mi diastolic) at 0.6cc LV volu was greater in Group (76.17±19.2 mmHg), than in Group II (52.0±25.21, p=0.021). Maximum +dP/dT at 0.6 cc LV vol was greater in Group I (854.47±381.8mmHg/sec) than in Group (485.10±284.14 mmHg/sec, p=0.025). Percent water of total ventric weight was 82.0% for Group I, 81.6% for Group II. Continuous perfu preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 2 yields left ventricular function is superior to 8-hr crystalloid perfus despite similar myocardial edema. Extended cardiac perfusion preserva with PEG-Hb may prove to be useful in transplantation.

DL Serna, LL Powell, WC Wallace, J West, C Kahwaji, G Cogen Smulowitz, E Steward, R Purdy, JC Milliken. UC Irvine.

To PEG-Hb solution was added KCl (4 mmol/L), Na^+ (145 mmol/L), $MgSO_4$ (5.1 mmol/L), $CaCl_2$ (0.4 mmol/L), 12.5 mg/L lidocaine, heparin (1250 units/L), dextrose (1.25gm/L), human albumin (1.6 gm/L), human insulin (3.1 units/L). PO_2 was maintained greater than 500 mmHg, and pH of 7.1 (37°C). Cardiac function was measured in the non-working state 2 hours after transfer to a standard crystalloid Langendorff circuit.

Results: Developed LV pressure at 0.5cc LV volume was similar in Group I (57.94±4.69 mmHg), and Group III (61.45±14.45 mmHg, p=0.71), but trended toward superiority compared to Group II (39.72±6.98 mmHg, p=0.32). Maximum –dp/dT at 0.5 cc LV volume was similar in Group I (–376.58±33.78 mmHg/sec), Group II (426.32±139.15 mmHg/sec, p=0.77), and Group III (–326.73±47.75 mmHg/sec, p=0.23). Percent water of total ventricular weight was 82.0% for Group I, 82.3% for Group II, and 81.5% for Group III.





Conclusions: Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 20°C yields left ventricular function that may be superior to 4 hrs of hypothermic storage and is similar to that of non-ischemic control hearts. Extended cardiac perfusion preservation with PEG-Hb may prove to be useful in transplantation.

Continuous Cardiac Perfusion Preservation with PEG-Hb Improves Perfomance over Standard Ischemic Hypothermic Storage

Dan L. Serna, Ledford L. Powell, Chadi I. Kahwaji, William C. Wallace, Peter Smulowitz, Justin West, Peter Connolly, Gerry Beckham, Earl Steward, Ralph Purdy, Jeffrey C. Milliken.

Cardiac preservation for transplantation is generally limited by ischemic hypothermic storage of 4 to 6 hours. Hypothermic perfusion preservation with an oxygen carrying hemoglobin solution may extend preservation times and decrease ischemic injury. The purpose of this study was to compare cardiac function after 8 hrs of continuous hypothermic perfusion with a PEG-Hemoglobin(Hb) solution to the clinical standard of hypothermic ischemic preservation.
Methods: The hearts of 28 anesthetized and intubuted NZW rabbits were harvested after cold cardioplegic arrest. Group I(n=10) hearts were perfused with a PEG-Hb (Enzon, Inc., Piscataway, NJ) solution at 20C and 30 mmHg for 8 hours. Group II(n=10) hearts were preserved by cold ischemic storage for 4 hours at 4C, and Group III(n=8) were tested immediately after harvest. LV function was measured in the non-working state 15 min after transfer to a standard crystalloid Langendorff circuit.
Results: Developed LV pressure at 0.5cc LV volume was greater in Group I (75.7±10.3mmHg) than Group II (49.1±5.4mmHg,p=.04) and similar to Group III (69.41±5.1mmHg, p=.6). Maximum-dp/dT at 0.5 cc LV volume was greater in Group I(-610.6±68.4mmHg/sec) than Group II (-354.8±49.1mmHg/sec,p=.01) and trended toward superiority over Group III(-456.2±44.1mmHg/sec,p=.09). Maximum +dp/dT at 0.5cc LV volume was greater in Group I (964.9±156.6mmHg/sec) than both Group II (428.4±54.9mmHg/sec,p=.004) and Group III(514.6±48.9mmHg/sec,p=.02).
Conclusions: Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb solution at 30 mmHg and 20 C yields left ventricular function that is superior to 4 hrs of ischemic hypothermic storage and is similar to that of fresh control hearts. Extended cardiac perfusion preservation with PEG-Hb deserves further investigation in large animal transplant models.

CARDIAC FUNCTION AFTER 8-HOUR STORAGE USING PEG-HEMOGLOBIN VS. CRYSTALLOID PERFUSION

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ABSTRACT

Introduction: Efforts to extend myocardial preservation for transplantation by crystalloid perfusion have been limited by edema and compromised function. We hypothesized that hypothermic perfusion preservation with a polyethylene glycol (PEG) conjugated hemoglobin solution may extend preservation times. The purpose of this study was to compare cardiac function after continuous perfusion using a hypocalcemic normokalemic crystalloid perfusate with and without the addition of PEG-Hemoglobin (Hb).

Methods: The hearts of 20 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=10) hearts were continuously perfused with a hypocalcemic, normokalemic 3% PEG-Hb solution at 20°C and 30 mmHg for 8 hours. Group II (n=10) hearts were continuously perfused with chemically similar crystalloid solution without PEG-Hb for 8 hours under identical conditions to Group I hearts. Cardiac function was measured with a left ventricular force transducer after transfer to a standard crystalloid Langendorff circuit at 37 °C and an aortic root pressure of 59 mmHg.

Results: After 8 hours of perfusion preservation, heart rate was similar for groups I and II (p=NS). Coronary blood flow after preservation and during preservation was similar between PEG-Hb and crystalloid preserved hearts (p=NS). Left ventricular developed pressure, peak dP/d*t*, and peak -dP/d*t* were superior in hearts preserved with PEG-Hb.

Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II (p=NS).

Conclusions: Continuous perfusion preservation of rabbit hearts for 8 hours with a hypocalcemic normokalemic PEG-Hb based solution at 30 mmHg and 20°C yields left ventricular function that is superior to crystalloid perfusion without the addition of PEG-Hb, despite similar myocardial edema and coronary flow. Extended cardiac perfusion preservation with this PEG-Hb based solution deserves further study.

INTRODUCTION

Heart failure affects more than 3 million patients in the United States (1). Almost one-third of these patients have New York Heart Association functional class III or IV heart failure, often characterized by progressive deterioration and frequent hospital admissions. Annual expenditures for heart failure have been estimated to be as high as $38 billion, of which $23 billion is for hospital stays and non-surgical treatment prior to transplantation (1). Federal legislation has recently been passed allowing the distribution of donor organs to recipient matches outside the geographic range of the donor. Meanwhile, existing techniques of preservation and donor organ distribution remain archaic. There is both a humanitarian and economic need to develop innovative techniques of donor heart procurement, preservation, and distribution.

The current method of donor heart preservation involves cold cardioplegic arrest and storage at near freezing temperatures. Because of ongoing ischemia, this preservation technique prohibits extended storage of donor organs, use of more efficacious methods of tissue typing, as well as delivery of donor hearts over large distances. The current preservation technique also leads to irreversible graft damage.

Preservation by continuous coronary artery perfusion allows for greater preservation times than hypothermic ischemic preservation (2). Continuous coronary artery perfusion allows for an ongoing supply of substrate as well as removal of metabolic waste products. Three general types of solution have been examined for their efficacy as cardiac preservation agents. Perfusion with crystalloid, cardioplegia-type solutions have shown limited promise (3-7). Perfusion preservation using these solutions has been limited by edema and compromised cardiac function (3-7). Similarly, studies examining perfluorocarbon emulsions as perfusion preservation media for the donor heart have produced mixed results (2,8-11). Further, perfluorochemicals are very expensive and have questionable safety profiles (8-10).

Hemoglobin-based blood substitutes have more recently been developed for use as blood replacements in trauma situations and surgery. Use of these solutions as organ preservation solutions may lengthen the window of *ex vivo* cardiac preservation with a concomitant decrease in ischemia. We hypothesized that hypothermic perfusion preservation with an hypocalcemic, normokalemic, polyethylene glycol conjugated hemoglobin (PEG-Hb) based solution over 8 hours would preserve left ventricular function above that obtained with a chemically identical crystalloid solution without PEG-Hb. We suspect that this PEG-Hb solution may optimize the donor heart during its preservation period. Moreover, optimization of perfusion preservation using this solution may, in time, allow considerable widening of the window of *ex vivo* cardiac preservation, allowing transportation of donor organs over large distances, more thorough tissue typing and matching, and as well as improved post-implantation graft function and survival.

The purpose of this study is to compare *ex vivo* adult rabbit heart preservation after continuous coronary artery perfusion using a hypocalcemic, normokalemic PEG-Hb solution versus an identical crystalloid solution not containing PEG-Hb. This work will lay the foundation for future investigation comparing perfusion preservation with PEG-Hb based solutions to hypothermic ischemic storage preservation as well as PEG-Hb solutions containing specific enhancers of myocardial preservation.

METHODS

All animals received humane care in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 86-23). The Institutional Animal Care and Use Committee of the University of California, Irvine, approved animal procedures.

Experimental Design

The hearts of 20 anesthetized and ventilated NZW rabbits were harvested after cold cardioplegic arrest. Group I (n=10) hearts were continuously perfused with a normokalemic hypocalcemic PEG-Hb based solution at 20°C and 30 mmHg of aortic root pressure for 8 hours. Group II (n=10) hearts were identically preserved with a crystalloid solution identical in composition to Group I hearts, but without the addition of PEG-Hb.

Cardiac Procurement

Twenty adult male New Zealand White rabbits (3 to 3.5 kg) were anesthetized using an intramuscular injection of 50mg ketamine and 5mg xylazine per kilogram. Lactated Ringers solution was infused through an IV catheter in a marginal ear vein at a rate of 5 to 15 cc/hr. The rabbits were mechanically ventilated using a Servo Animal

Ventilator (model #900C, Siemens-Elema, Sweden). Anesthesia was maintained with intravenous ketamine/xylazine in a 1:1 ratio. A median sternotomy followed by a longitudinal pericardial incision was performed, exposing the heart and mediastinal vessels. All rabbits received 1,000 U heparin sodium/kg IV. The innominate artery, the aortic arch between the brachiocephalic trunk and left carotid artery, as well as the inferior and superior vena cava were the identified and isolated. Upon ligation of the inferior and superior vena cava, the innominate artery was cannulated using an 18 Ga angiocatheter. 60 cc of hypothermic cardioplegia solution (2-4°C) was administered to the coronary arteries via the innominate artery over 3 minutes. An arteriotomy was made in the pulmonary trunk to decompress the right ventricle. Hypothermic normal saline (2-4°C) was used to cool the heart during cardioplegia infusion. The heart was quickly excised and placed in cold saline (4°C) for further dissection. The heart was trimmed of excess soft tissue including lungs, trachea, and thymus. All hearts were placed onto the preservation circuit by cannulation at the ascending aorta. Coronary perfusion was begun within 5 minutes of cardiectomy. All hearts were preserved for 8 hours by continuous coronary artery perfusion. Aortic root pressure was maintained at 30 mmHg. Temperature of the perfusate was maintained at 20°C. All hearts were perfused and immersed in the respective preservation solutions for the entire 8-hour preservation period. 95%O_2/5%CO_2 administration was begun using the membrane oxygenator 15 minutes after transfer of the heart to the preservation circuit. PaO_2 was maintained at a level greater than or equal to 600mHg. The preservation circuit consisted of a centrifugal pump (Medtronic Bio-Medicus pumphead, Model # 9154R, Medtronic Blood Systems, Inc., Anaheim, CA) and Bio-Console (Medtronic Bio-Medicus

Inc., Eden Prairie, MN), an adult membrane oxygenator (Sarns/3M Health Care, Inc., Ann Arbor, MI), C-Flex Consolidated Polymer tubing (Fischer, Largo, FL), a 40 um blood filter (Pall Biomedical, Inc, Fajardo, PR), and 2 glass reservoirs. The temperature of perfusate was maintained by a heater/cooler (Fisher Scientific Inc., Pittsburgh, PA) which was circulated through the membrane oxygenator (figure 1).

Preservation Solutions

The composition of the PEG-Hb based preservation fluids is as follows: 3% PEG-Hb, KCL (4.7 mEq/L), NaCl (148.7 mmol/L), NaPO4 (2.5 mmol/L), NaHCO3 (2.5 mmol/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6units/L), Tromethamine (THAM) solution (7.3 cc/L).

The composition of the crystalloid preservation solution is as follows: KCL (4.7 mEq/L), NaCl (150.7 mEq/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (1.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human albumin (1.5 gm/L), human insulin (30.6 units/L), and Tromethamine (THAM) solution (7.3 cc/L).

Postpreservation Assessment of Cardiac Function:

At the end of the 8-hour preservation period, all hearts were transferred to an isolated heart perfusion apparatus for purposes of data collection. Coronary perfusion via the aortic root was immediately begun at 37°C and 59 mmHg aortic root pressure. $95\%O_2/5\%CO_2$ administration was begun using the membrane oxygenator 15 minutes after transfer of the heart to this circuit. PaO_2 was maintained at a level greater than or equal to 600mHg. After 15 minutes of coronary perfusion in this position, coronary flow, heart rate, left ventricular developed pressure (LVP), peak dP/dt, and peak $-dP/dt$ were

measured. Coronary flow and heart rate were measured every 15 minutes for 2 hours. LVP, peak dP/d*t*, and peak –dP/d*t* were measured again at 75 and 135 minutes following transfer to the second circuit. Heart rate was measured by counting left ventricular contractions over the course of one minute. Coronary flow was measured by collecting the effluent that exited from the pulmonary artery over course of one minute. LVP, peak dP/d*t*, and peak –dP/d*t* were measured in the beating, nonworking position during continuous coronary artery perfusion. Developed left ventricular pressure (systolic minus diastolic) and peak rates of left ventricular pressure development (dP/dt_{max}) and relaxation ($-dP/dt_{max}$) were measured using a left ventricular force transducer (Biopac Systems, Inc., Santa Barbara, CA). Data from the LV force transducer was digitized using an analog to digital converter (Biopac Systems, Inc., Santa Barbara, CA) and analyzed using Acknowledge software (Version 3.2.6, Biopac Systems, Inc., Santa Barbara, CA) and a desktop computer (Nexstar, Fremont, CA). The testing circuit consisted of a centrifugal pump (Medtronic Bio-Medicus pumphead, Model # 9154R, Medtronic Blood Systems, Inc., Anaheim, CA) and Bio-Console (Medtronic Bio-Medicus Inc., Eden Prairie, MN), an adult membrane oxygenator (Sarns/3M Health Care, Inc., Ann Arbor, MI), C-Flex Consolidated Polymer tubing (Fischer, Largo, FL), a 40 um blood filter (Pall Biomedical, Inc, Fajardo, PR), and 2 glass reservoirs. The temperature of perfusate was maintained by a heater/cooler (Fisher Scientific Inc., Pittsburgh, PA) which was circulated through the membrane oxygenator.

Testing Solution: Left ventricular function was assessed in all hearts in both groups using a standard physiologic crystalloid solution. The composition was as follows: KCL

(4.7 mEq/L), NaCl (151.5 mEq/L), $MgSO_4$ (5.0 mEq/L), $CaCl_2$ (2.0 mEq/L), lidocaine HCl (12.5 mg/L), heparin sodium (1250 units/L), dextrose (6.1 mOsm/L), human insulin (30.7 units/L), and Tromethamine (THAM) solution (6.1 cc/L).

Measurement of ventricular water content: After 135 minutes of retrograde aortic perfusion on the testing circuit, the ventricular myocardium of the initial 5 hearts in each group was dissected free of atria and other soft tissue. The left ventricular myocardium was weighed before and after desiccation at 110°C.

Data Analysis

Data are reported as means ± SE. Statistical analysis was performed using Systal 7.0.1 software package (SPSS, Inc., Chicago, IL). A p value of less than 0.05 was considered significant.

Materials

Bovine PEG-Hb was obtained from Enzon, Inc. (Piscataway, NJ) in a solution containing 6% PEG-Hb, 5 mM NaPO4, 5 mM NaHCO3, and 150 mM NaCl. Normal saline solution (0.9% NaCl) was obtained from Baxter Health Care (Irvine, CA). Solutions were monitored using a blood gas analyzer (288 Blood Gas System, Ciba-Corning Diagnostics Corp., Medfield, MA), an Automated Coagulation Timer (Medtronic Hemotec, Inc., Englewood, CO) and a blood glucose meter (Lifescan, Inc., Milpitas, CA). Membrane oxygenators were obtained from Sarns/3M Health Care, Inc. (Ann Arbor, MI).

RESULTS

Developed LV pressure: Developed LV pressure at 0.5 cc LV volume was similar between PEG-Hb and crystalloid preserved hearts at 15 minutes after the end of

preservation ($p=0.46$, figure 2). However, developed LV pressure at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 75 ($p=0.006$) and 135 minutes ($p=0.002$) after the end of preservation.

Maximum rate of LV contraction: Peak dP/dt_{max} at 0.5 cc LV volume trended toward superiority amongst hearts preserved using PEG-Hb solution compared to crystalloid preserved hearts, at 15 minutes after the end of preservation ($p=0.10$, figure 3). However, peak dP/dt_{max} at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 75 ($p=0.01$) and 135 minutes ($p=0.001$) after the end of preservation.

Maximum rate of LV relaxation: Peak $-dP/dt_{max}$ at 0.5 cc LV volume was similar between PEG-Hb and crystalloid preserved hearts at 15 minutes ($p=0.27$) after the end of preservation and trended toward superiority at 75 minutes after the end of preservation ($p=0.07$, figure 4). Peak $-dP/dt_{max}$ at 0.5 cc LV volume was superior in PEG-Hb preserved hearts compared to crystalloid preserved hearts at 135 minutes ($p=0.006$) after the end of preservation.

Ventricular Water Content: Percent water of total ventricular weight was 82.0% for Group I, 81.6% for Group II (p=NS).

Coronary Flow: Coronary flow after preservation was similar between PEG-Hb and crystalloid preserved hearts (figure 5).

Heart Rate: Heart rate was the same for group I and II through the testing period (p=NS, Table 1).

CONCLUSIONS

There are two general techniques of *ex vivo* cardiac preservation for transplantation. The standard of care and currently used technique is hypothermic ischemic immersion storage of the donor cardiac allograft. The second method of cardiac preservation is coronary perfusion preservation. These two methods can and have been used in combination with improved results. Perfusion preservation is superior to immersion preservation as it allows the continuous washout of metabolic waste products, as well as delivery of nutrients, metabolic substrate, and depending upon the solution used, oxygen to the myocardium (2). For several reasons, perfusion preservation has not been applied clinically to *ex vivo* cardiac preservation for transplantation. First, a user friendly, practical, and portable perfusion preservation device is not currently available. Second, most research into perfusion preservation to date has been performed using saline-based cardioplegia solutions and perfluorocarbons. Saline-based cardioplegia solutions, unfortunately, carry very little oxygen and hence their use is associated with considerable ischemic injury to the donor organ (3-7). Perfluorocarbon based solutions have demonstrated mixed results for the purpose of cardiac preservation, are extremely expensive and possibly toxic (2,8-11).

Perfusion preservation using stroma-free hemoglobin based solutions represents an innovative means of *ex vivo* cardiac preservation. Stroma-free hemoglobins were initially developed as blood substitutes for use in the treatment of life threatening hemorrhage secondary to trauma. There is strong interest amongst transplant scientists in the potential for these solutions as organ preservation solutions. The purpose of this

study was to assess the utility of perfusion preservation using normokalemic hypocalcemic polyethylene glycol coated bovine hemoglobin based solution.

The covalent attachment of PEG to the Hb gives the Hb entity a bigger particle size, which may help to maintain the hemoglobin within the vascular space by making it too large to easily migrate into the interstitial space. One question that arises is whether the mechanism of benefit in this study is secondary to the PEG or the hemoglobin or the combination of PEG with hemoblobin? There exists theoretical basis for a beneficial action by both PEG as well as hemoglobin when each is used alone. When added to Bretschneider's HTK cardioplegic solution, PEG is associated with improved recovery of left ventricular function as well as less myocardial edema (12). The mechanism of action of PEG is uncertain, but probably includes prevention of osmotic swelling and suppression of lipid peroxidation (12). Hemoglobin, meanwhile, is capable of carrying and delivering oxygen to the myocardiocytes. Similarly, hemoglobin likely contributes to the oncotic effect of the solution, acting to minimize tissue edema.

We made the preservation solution hypocalcemic because the intracellular accumulation of calcium during ischemia and reperfusion is associated with cellular injury (13-17) and a hypoxically stressed heart may be protected by a hypocalcemic solution (13). We made the solution normokalemic since we suspected that a beating heart would be less susceptible to edema formation in the setting of adequate oxygen delivery. Finally, we made the preservation solution slightly hypermagnesemic because magnesium inhibits the membrane transport of calcium, and thus intracellular accumulation of calcium, which should help to prevent the deleterious effects of calcium (18-21). Magnesium has been shown to attenuate deleterious effects of calcium in

ischemic piglet hearts, which are more sensitive to the detrimental effects of calcium than are adult hearts (22).

This study utilized bovine hemoglobin, which is similar in structure to human hemoglobin but possess several important differences. First, the oxygen-hemoglobin dissociation curve is slightly shifted such that bovine hemoglobin more easily releases oxygen at the tissue level. Second, while 2,3-diphosphoglycerate decreases the affinity of human hemoglobin for oxygen, chloride anion similarly binds to and affects the affinity of bovine hemoglobin for oxygen, increasing the dissociation of oxygen from bovine hemoglobin at the tissue level. PEG-modified Hb, in a formulation different from that described in this manuscript, is currently being investigated in phase II clinical trials for use as an enhancer to radiation therapy in patients with cancer.

There is tremendous value to lengthening the window of cardiac preservation. First, less ischemia to the donor organ improves posttransplant graft function and recipient survival. Second, lengthening the window of cardiac preservation will allow prospective HLA matching as well as the transport of hearts over greater distances to better matched recipients. HLA mismatching results in an increased risk of early high-grade rejection. This results in rehospitalization and an increased use of resources. Coronary artery vasculopathy (CAV) is related to the degree of HLA mismatching. There is increased CAV among patients with more rejection episodes (23). HLA-DR mismatching has been shown to have strong adverse effects on graft survival when examined for up to 10 years (24-25).

This solution should be tested in the preservation of other organs as well. The heart is the most metabolically active and the most sensitive to ischemia of the

commonly transplanted solid organs. This data suggests PEG-Hb based solutions should be useful is the perfusion preservation of other solid transplantable organs. This suspicion should be investigated with controlled animal experiments. Future investigation will also be useful in the optimization of these Hb based solutions. Some questions that remain include the optimal preservation temperature, Hb concentration, as well as the optimal potassium concentration.

Continuous perfusion preservation of rabbit hearts for 8 hrs with PEG-Hb at 30 mmHg and 20^0C yields left ventricular function that is superior to 8-hr perfusion with a chemically similar crystalloid solution without addition of PEG-Hb, despite similar myocardial edema. Extended cardiac perfusion preservation with PEG-Hb may prove to be useful in transplantation. The next line of investigation should compare perfusion preservation with fresh hearts and with hearts that have been preserved using techniques similar to the standard of care. Furthermore, preserved hearts should be transplanted to evaluate left ventricular function after reperfusion with blood.

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LEGEND

Figure 1. Isolated heart perfusion preservation circuit.

Figure 2. Developed LV pressure at 15, 75, and 135 minutes after preservation.

Figure 3. Maximum rate of LV contraction at 15, 75, and 135 minutes after preservation.

Figure 4. Maximum rate of LV relaxation at 15, 75, and 135 minutes after preservation.

Figure 5. Coronary blood flow after 8 hours of perfusion preservation.

Table 1. Heart rate after 8 hours of perfusion preservation.

	Heart Rate		
Time (minutes)	PEG-Hb	Crystalloid	p value
15	117±8.4	100.8±8.4	0.19
30	104.9±7.9	98.4±4.9	0.48
45	98.8±6.6	95.2±4.6	0.66
60	94.1±6.6	97.6±5.7	0.70
75	90.9±6.8	96.2±5.5	0.55
90	99.2±6.6	97.0±3.5	0.77
105	97.8±6.3	89.1±4.8	0.30
120	95.0±6.4	75.8±11.5	0.14
135	100.5±6.3	73.9±13.7	0.07

Table 1.